Author's manuscript. Final version published as: Rachek LI, Grishko VI, Musiyenko SI, Kelley MR, LeDoux SP, Wilson GL. Conditional targeting of the DNA repair enzyme hOGG1 into mitochondria. J Biol Chem. 2002 Nov 22;277(47):44932-7. Available from: http://dx.doi.org/10.1074/jbc.M208770200

Conditional Targeting of the DNA Repair Enzyme hOGG1 into Mitochondria*

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Running title: Conditional targeting of hOGG1 and mitochondrial DNA repair

* This research was supported by National Institutes of Health Grants ES03456, ES05865 and AG19602, and a Feasibility Grant from the United States Army. The costs of publication of this article were defrayed in part by the payment of page charges. Therefore, this article must be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1]The abbreviations used are: ROS, reactive oxygen species; mtDNA, mitochondrial DNA; BER, base excision repair; OGG1, 8-oxoguanine DNA glycosylase/apurinic lyase; MTS, mitochondrial targeting sequence; AP, apurinic/apyrimidinic; MnSOD, manganese superoxide dismutase; Tet, tetracycline; Dox, doxycycline; GFP, green fluorescent protein; Luc, luciferase; HBSS, Hank's balanced salt solution; mtODE, mitochondrial Oxidative Damage Endonuclease.

SUMMARY

Oxidative damage to mitochondrial DNA (mtDNA) has been suggested to be a key factor in the etiologies of many diseases and in the normal process of aging. Although the presence of a repair system to remove this damage has been demonstrated, the mechanisms involved in this repair have not been well-defined. In an effort to better understand the physiological role of recombinant OGG1 in mtDNA repair, we

constructed an expression vector, containing the gene for OGG1 downstream of the mitochondrial localization sequence from MnSOD. This gene construct was placed under the control of a Tet-regulated promoter. Transfected cells that conditionally expressed OGG1 in the absence of the tetracycline analogue doxycycline and targeted this recombinant protein to mitochondria were generated. Western blots of mitochondrial extracts, from vector and OGG1 transfected clones with and without doxycycline, revealed that removal of doxycycline for 4 days caused an approximate eight-fold increase in the amount of OGG1 protein in mitochondria. Enzyme activity assays and DNA repair studies showed that the doxycycline-dependant recombinant OGG1 is functional. Functional studies revealed that cells containing recombinant OGG1 were more proficient at repairing oxidative damage in their mtDNA, and this increased repair led to increased cellular survival following oxidative stress**.**

INTRODUCTION

The importance of mitochondrial DNA (mtDNA) for cellular integrity and function is underscored by the fact that a variety of diseases have been associated with mutations in mtDNA, including diabetes (1, 2) ischemic heart disease (3), Parkinson's disease (4-6), Alzheimer's disease (7-9), and the normal process of aging (10-12). The damage to mtDNA which leads to these mutations likely results from exposure to reactive oxygen species (ROS). These noxious agents are formed continuously in the mitochondria by electron leakage from the respiratory chains. Although oxidative phosphorylation in mitochondria is essential for producing the energy that is required to sustain life, approximately one to two percent of the total oxygen processed by the electron transport chains is reduced via one electron reduction to form ROS such as superoxide. Therefore, mtDNA which is located near the electron transport chains is continuously bombarded with these noxious agents. Although there are substantial antioxidant defenses in mitochondria to protect against the deleterious effects of ROS, damage to mtDNA still occurs. Therefore, efficient repair mechanisms are imperative to prevent these lesions from becoming permanent mutations.

Over the past decade, there have been substantial advances in the understanding of the mechanisms involved in the repair of oxidative damage in mitochondrial DNA (mtDNA). Work from our laboratory and that of others has established that repair of this damage is via a base excision repair (BER) pathway (13-16). In support of this notion is the finding that mitochondria contain the basic enzymes required for BER (17, 18), including the glycosylase/AP lyase OGG1 which is necessary for the initial steps in the removal of the mutagenic lesion 8-oxoguanine. Because of its lyase activity, this enzyme also may play a role in cleaning the 3' end of oxidative lesions to the sugar-phosphate backbone. The human OGG1 gene encodes two major isoforms: α-hOGG1 and β-hOGG1, resulting from alternative splicing of the transcript (19-21). Both forms have a putative mitochondrial localization signal and only α-hOGG1 has a nuclear localization signal (21). Recent studies have shown that β-hOGG1 is targeted to mitochondria. The importance of the Ogg1 DNA glycosylase in the repair of oxidative damage was shown in Ogg1-deficient mice. These animals accumulate abnormally high levels of 8-OxoG in their genomes (22). Furthermore, no cleavage of 8-oxoG:C containing substrate was detected in tissue extracts from Ogg1 knockout mice, indicating that OGG1 is the only mammalian glycosylase that can efficiently remove 8-OxoG from 8-oxoG:C pairs. These findings correlate well with previous data on the substrate specificity of the murine and human OGG1 enzyme (23, 24). Although extracts of Ogg1 null mouse tissues were not able to excise the damaged base *in vitro*, significant slow release of the adducted base was observed from proliferating cells *in vivo*, suggesting that there is an alternative repair pathway in those cells (22). More recently, studies were reported by Bohr et al. (25) that used Ogg1 null mice to show that OGG1 is more important in mitochondrial than in nuclear DNA repair of oxidative damage (22). Based on these studies, it is likely that OGG1 is the only glycosylase for 8-OxoG removal in mouse mitochondria. Additionally, it was found that mitochondrial OGG1 activity increased with age in the mouse liver (26), whereas the nuclear OGG1 activity decreased slightly over the same time interval. This differential change

with age in the mitochondrial and nuclear 8-OxoG glycosylase activities suggests that the expression of these two isoforms may be differentially regulated. When considered together, these findings indicate that BER initiated by OGG1 is an essential enzyme required for protection of mtDNA against oxidative damage.

Previous studies from our laboratory have shown that mitochondrial DNA repair and cellular survival can be enhanced by targeting recombinant OGG1 to mitochondria (27). However, to fully evaluate the potential of using recombinant repair enzymes to alter mtDNA repair, it would be very beneficial to have their expression under the control of an inducible promoter. The tetracycline (Tet)–regulatable expression system using control elements of the tetracycline resistance operon encoded in Tn10 of *E. coli* (28) has shown promise for conditional gene expression. This system relies on the presence or absence of tetracycline (Tet) or a commonly used analog, doxycyclin (Dox), to control gene expression. For this study, we constructed a vector containing the sequence for hOGG1 downstream of the MnSOD mitochondrial targeting sequence under the control of the Tet-response element and introduced it into HeLa cells previously transfected with pTet-Off plasmid. When hOGG1 was conditionally expressed, we investigated the level and location of the recombinant protein and its effect on mtDNA repair and cell survival following exposure to ROS. The results demonstrate that DNA repair proteins can be conditionally expressed under the control of an inducible promoter and targeted to mitochondria. The Tet-dependent overexpression of OGG1 causes enhanced repair of oxidative damage to mtDNA and increased cellular resistance to the lethal effects of ROS.

EXPERIMENTAL PROCEDURES

The OGG1 Construct-An *Eco*RI –*Xho*I fragment containing the MTS and the OGG1 coding region (27) was filled in with Klenow DNA polymerase and ligated into the *Pvu*II restriction site of the pTRE2hyg expression vector (CLONTECH Laboratories, Inc.), which contained a tetracycline-responsive element. The construct obtained was sequenced to verify the integrity of the reading frame and the fidelity of the sequence.

Cell Culture and Transfection -HeLa cells were obtained from American Type Culture Collection. The cells were transfected with pTet-Off plasmid (CLONTECH Laboratories, Inc.) using Fugene 6 reagent (Roche Molecular Biochemicals) according to the manufacturer's recommendations. Stable transfectants were selected in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (CLONTECH Laboratories, Inc.), 2 mM L-glutamine (Life Technologies, Inc), 800 μg/ml G418 (Geneticin) and 50 μg/ml penicillin/streptomycin (Sigma). Integration of the pTet-Off plasmid was confirmed by PCR and expression of the Tet-dependent transactivator (tTa) was tested using the Luciferase Assay System according to the manufacturers instructions (Promega) in the presence or absence of 1 μg/ml doxycyclin hydrocloride (Dox, Sigma). Transient transfections with pTRE2hyg/Luc (CLONTECH Laboratories, Inc.) were performed using Polyfect Transfection Reagent (Qiagen) following

the manufacturers instructions. The plasmids pcDNA3.1/His/LacZ (Invitrogen) and pEGFP-N3 (CLONTECH Laboratories, Inc.) containing β-galactosidase or GFP genes under the control of constitutive promoters, respectively, were used as reporter plasmids. The transfection efficiencies were usually >30 %, as determined by control transfection with pEGFP-N3. Cells were harvested 48-72 h after transfection and luciferase activities were assayed. For normalization of transfection efficiency, a control LacZ expression vector (pcDNA3.1/His/LacZ) was used. Luciferase (Luc) values were normalized to β-galactosidase activities by performing chlorophenol red β-D-galactosidase (CPRG) -assays (29). Enzyme activities were adjusted to the protein content of the cell lysates, which was determined by using the Bio-Rad protein dye micro-assay according to the manufacturer's recommendations (Bio-Rad, Hercules, CA). The clone of HeLa Tet-Off that expressed the lowest basal level of Luc in the presence of Dox and the highest induced level of Luc activity in the absence of the antibiotic was selected for the second transfection with pTRE2hyg/MTS-OGG1 plasmid. A pTRE2hyg vector without the insert was used to transfect Tet-Off HeLa cells as a control. After this second transfection, the colonies were grown in DMEM with 800 μg/ml G418 and 250 μg/ml hygromycin B (Life Technologies, Inc.) and the surviving ones were tested for the integration of MTS-OGG1 insert by Southern hybridization**.** The selected Tet-Off/MTS-OGG1 clones were maintained in DMEM (Life Technologies, Inc.), supplemented with 10% fetal bovine serum (CLONTECH Laboratories, Inc.), 800 μg/ml G418 (Geneticin), 200 μg/ml hygromycin B and with (Dox+) or without (Dox-) 1μg/ml doxycyclin. Doxycyclin was withdrawn from the media of Dox- maintained clones 4 days before any experiment.

Preparation of Mitochondrial Fractions - One 150 mm dish of each cell type (MTS-OGG1 and control vector transfected cells) at confluence was harvested and treated with ice-cold digitonin (325 mM digitonin, 2.5 mM EDTA, 250 mM mannitol, and 17 mM MOPS, pH 7.4) for 80 s. The lysed cells were then added to mannitol-sucrose buffer to a final strength of 1X (210 mM mannitol, 70 mM sucrose, 5 mM EDTA, 5 mMTris, pH 7.5). The suspension was then centrifuged for 10 min at 800 g at 4^{o}C to pellet nuclei. The supernatant was centrifuged two more times for 10 min at 800 g at 4^{o}C to get rid of nuclear debris. The mitochondrial fraction was pelleted by centrifugation at 20,000 g at 4^{o}C for 20 min. Isolated mitochondria were suspended in a buffer of 20 mM HEPES, pH 7.6; 1 mM EDTA; 5 mM DTT; 300 mM KCl; 5% glycerol; and 5 μl of protease inhibitors mixture (Sigma, St. Louis, MO) per ml. These suspensions were briefly sonicated on ice, centrifuged once more at 5,000 g to pellet any remaining debris, and the supernatant protein was used for Western blot assays. The protein concentration was determined using the Bio-Rad protein dye micro-assay according to the manufacturer's recommendation (Bio-Rad, Hercules, CA).

Western Blot Analysis - SDS-polyacrylamide gel electrophoresis and transfer of separated proteins to PVDF-membrane were performed as previously described (27) with some minor modifications. Blocking and antibody immunoblotting were performed in 5% nonfat dry milk and Tris-buffered saline

with 0.1% Tween 20 (TBS-T). TBS-T and TBS were used for washing. The polyclonal anti-hOGG1 antibodies were from Novus Biologicals (Littleton, CO); anti-cytochrome *c* monoclonal antibody was purchased from PharMingen (San Diego, CA). Complexes formed by these antibodies were detected with horse radish peroxidase conjugated anti-mouse IgG or anti-rabbit IgG antibodies (Promega, Madison, WI) using chemiluminescent reagents (SuperSignal, Pierce, Rockford, IL).

OGG Activity Assays - A 24 -mer oligonucleotide with 8-oxoguanine at the 10^{th} position (Trevigen) was end-labeled. The labeling reaction contained 5 pmol of single-strand 8-oxoguanine oligonucleotide, 5 pmol of γ-^{32} P, T4 polynucleotide kinase, and appropriate kinase buffer in a total volume of 20 µl (incubation for 30 min at 37^{o} C followed by 2 min at 90^{o} C). Complementary oligonucleotide (5 pmol) was then added at room temperature to form duplex DNA. Equal amounts of protein from mitochondrial fractions isolated from transfected cells grown in media with or without Dox were used in assays with the labeled duplex oligonucleotide. Activity assays contained 0.2 pmol of labeled duplex oligonucleotide , 3 µl of 10XREC buffer (100 mM HEPES, pH 7.4, 1M KCl, 100 mM EDTA, and 1 mg/ml BSA) and organelle extract or 5 U of control formamidopyrimidine DNA glycosylase enzyme (Trevigen) in a total volume of 20 µl. Reaction mix was incubated for 3 and 6 h at 37^{o} C. Formamide/ bromphenol (80%/0.2%) dye was added to the mix and reaction products were resolved in 20% acrylamide, 8 M urea gels.

Drug Preparation and Exposure - For oxidative damage and repair experiments, cells were grown in 100 mm dishes for four days in media with or without Dox until 70-80% confluence. Menadione sodium bisulfite (2-methyl-1, 4-naphthoquinone sodium bisulfite, Sigma, St.Louis, MO) was dissolved in Hanks' balanced salt solution (HBSS) at a concentration of 800 µM for DNA repair experiments. Cells were rinsed with HBSS and then exposed to the appropriate amount of menadione for 20 min in a 37^{o}C, 5% CO_2 incubator. Control cultures were exposed to HBSS under the same conditions. After a 20 min exposure, cells were rinsed and lysed immediately (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% SDS, and 0.3 mg/ml proteinase K), or were allowed to repair for 2 or 6 h in regular growth media with or without Dox.

Quantitative and Neutral Southern Blots - MTS-OGG transfected and vector-only transfected cells were exposed to menadione as described above and cell lysates were incubated overnight at 37^{o}C. After addition of NaCl to a final concentration of 1 M, high molecular weight DNA was extracted with an equal volume of chloroform:isoamyl alcohol (24:1), precipitated with ammonium acetate and 2 volumes of cold ethanol and resuspended in water. DNA samples were digested with restriction endonuclease *Xho*I (10 units/µg of DNA) and, at the same time, treated with DNAse-free- RNase (~1.0 µg/ml) for 12-16 h at 37^{o}C. After digestion, samples were precipitated as before, resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) and precisely quantified using a Hoefer TKO 100 Mini-Fluorometer and TKO standards kit (Hoefer Scientific Instruments, San Francisco, CA). Samples containing 5 µg DNA were heated at

65^{o}C for 20 min and then cooled at room temperature for an additional 20 min. A sodium hydroxide solution then was added to a final concentration of 0.1 N and samples were incubated for 15 min at 37^{o}C. This produced single strand breaks at any abasic or sugar-modified site in the DNA. Next, samples were combined with 5 µl of loading dye, loaded onto a 0.6% alkaline agarose gel and electrophoresed at 30 Volts (1.5 Volts/ cm gel length) for approximately 16 h in an alkaline buffer consisting of 23 mM NaOH, 1 mM EDTA. The gels were stained with ethidium bromide to confirm equal loading. After standard gel washing, the DNA was transferred to a Zeta-Probe GT nylon membrane (BioRad, Hercules, CA). The membranes were cross-linked and hybridized with a ^{32}P-labeled human mtDNA-specific PCR-generated probe. Hybridization and subsequent washes were performed according to the manufacturer's recommendations. DNA damage and repair were determined as described previously (30). Neutral Southern blots were performed the same way, except that there was no alkaline pretreatment of samples and NaOH was not included in the loading dye, the 0.6% agarose gel, or the electrophoresis buffer. To confirm integration into the genome of the pTRE2hyg/MTS-OGG1 plasmid, DNA samples were digested with *Bam*HI and *Hind*III, and hybridization was performed with a ^{32}P-labeled MTS-OGG1 fragment generated using the RadPrime Labelling System (Life Technologies, Inc.).

Clonogenic Assays - Cells were counted with a hemocytometer and 250 cells were plated in each 60 mm dish to achieve sparse distribution. These cells were incubated for 24 h in normal culture medium to allow them to adhere to the culture vessel. Next, they were exposed to menadione exactly as described above, with the exception that lower concentrations of the drug were used due to the higher sensitivity of cells to oxidative stress when plated at the low density required for the clonogenic assay. For each cell type, controls (no menadione) and cultures receiving various concentrations of menadione were studied in triplicate. After 20 min of treatment, the menadione solution was replaced with normal culture medium (with or without Dox) and cells were grown for 10 days. Then colonies formed were stained with hematoxylin and counted.

Statistical Analysis – The data are presented as the means $\pm$ standard errors of three independent experiments. Data were compared with the use of two-way ANOVA followed by Bonferroni analysis. Statistical significance was determined at the 0.001 level.

RESULTS

Generation of Double Transfected Cell Lines – To fully evaluate the potential of using recombinant hOGG1 to alter mtDNA repair, we used HeLa cells transfected with MTS-OGG1 under the control of a Tet-mediated promoter. Initially, HeLa cells were stably transfected with the pTet-Off regulator plasmid. Following two weeks of selection, individual clones were isolated and the integration of the tTa activator was confirmed by PCR amplification. Expression of tTa was checked by Luc-assay after transient transfection of Tet-Off clones with a reporter plasmid pTRE2hyg/Luc. Selected clones were

grown in the absence and presence of Dox. Clones that expressed the lowest basal level of luciferase and showed the highest induced level (> 50 fold induction) of Luc-activity after Dox removal were chosen for the second transfection with the pTRE2hyg/MTS-OGG1 construct or control vector (pTRE2hyg).

Double transfected clones were selected in hygromycin and grown in the presence of Dox. DNA was isolated from cells transfected with MTS-OGG1 or vector only, and the presence of the insert was confirmed in the MTS-OGG1 transfected clones by hybridization with a 1.1 kb fragment containing a partial MTS-OGG1 sequence (Fig. 1). A clone of HeLa Tet-Off/MTS-OGG1-1 was chosen for subsequent study because it exhibited the highest level of MTS-OGG1 incorporation into its genome and produced the greatest amount of OGG1 protein in mitochondria after induction (Fig. 2).

Dox-Dependant Overexpression of OGG1 in Mitochondria - In order to show that the recombinant OGG1 enzyme was targeted directly to mitochondria and its expression was regulated by Dox, mitochondrial extracts were isolated from MTS-OGG1 and vector only transfected clones which were grown in media with or without Dox. Mitochondria were isolated by differential centrifugation and western blots were performed utilizing a polyclonal antibody to human OGG1. Even loading was confirmed by Coomassie Blue staining. The Tet-Off/MTS-OGG1-1 Dox- clone showed an increase in OGG1 protein (39 kDA) when compared to Dox+ Tet-Off/MTS-OGG1 repressed cells or vector-transfected cells (Fig. 2). This data confirmed that the recombinant OGG1 enzyme was targeted to mitochondria, and that OGG1 gene expression was regulated by Dox.

Dox-Dependant OGG1 Activity in Mitochondria - To determine whether the recombinant OGG1 was functional in mitochondria, OGG1-activity assays were performed. A labeled 24 - base pair duplex oligonucleotide with 8-oxoguanine placed at the 10^{th} position on one strand was incubated with mitochondrial extracts isolated from MTS-OGG1 and vector-transfected cells. As a positive control, the bacterial glycosylase/AP-lyase FPG (Trevigen) was used. As shown in Fig. 3, the mitochondrial extracts isolated from MTS-OGG1 (Dox-) clones were better able to cleave the DNA substrate than were the same cells grown in the presence of Dox and vector only transfected cells. Thus, this data reveals that the conditionally expressed recombinant OGG1 protein, which is targeted to mitochondria, is indeed functional.

Dox-Mediated Overexpression of OGG1 in Mitochondria and mtDNA Repair - To test whether the conditional overexpression of recombinant hOGG1 in the mitochondria has an effect on mtDNA repair capacity, an analysis of DNA repair was performed. To detect mtDNA damage and repair a quantitative Southern blot technique was used as described in the “Experimental Procedures”. First, dose response experiments were performed using different concentrations of menadione, which redox cycles with complex I of the electron transport chains in mitochondria and forms superoxide radical (31). A concentration of 800 μM menadione was chosen for the repair experiments because it produced an appropriate amount of lesions (~1 lesion per 10^4 normal nucleotides). There was no significant difference

in the initial break frequencies between clones which were grown in the absence or presence of Dox. Repair experiments were performed in which the MTS-OGG1 and vector transfectants were exposed to 800 μM menadione for 20 min and either lysed immediately or allowed to repair for 2 or 6 h in normal culture media with or without Dox. Control cells were incubated in drug diluent only. Total DNA was isolated from the lysed cells and quantitative Southern blot hybridizations were performed. As shown in Fig. 4A, the MTS-OGG1 (Dox-) clones repaired most of the damage within the initial 6 h following drug removal, whereas the vector transfectants and MTS-OGG1 (Dox+) clones did not repair an appreciable amount of the damage to their mtDNA. The average amount of repair from three separate experiments for each cell type is shown in Fig. 4B. These results document that recombinant hOGG1, when conditionally overexpressed and targeted to mitochondria, significantly enhances mtDNA repair of oxidative damage.

The Effect of Conditional Overexpression of OGG1 in Mitochondria on Cellular Viability - To determine whether the increase in mtDNA repair enhanced cellular survival following oxidative stress, a clonogenic assay was performed. Because sparsely growing cells are more sensitive to menadione than confluent monolayers, for these clonogenic studies we used lower doses of menadione than were required for the DNA repair experiments. Drug exposure and cellular growth in the presence or absence of Dox was performed using techniques described in the "Experimental Procedures" section. The colonies formed from cells that survived and proliferated following oxidative stress were counted after 10 days of culture. Fig. 5 shows that there is a significant enhancement in survival in the MTS-OGG1 (Dox-) transfectants. These viability data establish that conditional transduction of OGG1 in mitochondria leads to increased cellular survival after an oxidative challenge.

DISCUSSION

To our knowledge, this is the first report to show conditional expression of a recombinant DNA repair enzyme which is targeted to mitochondria. Previous studies by our laboratory showed that when a vector containing the gene for hOGG1 fused to the localization sequence from MnSOD was stably transfected into HeLa cells, repair of oxidative damage in mtDNA was increased and cellular survival enhanced (27). Although this work provided some important new information concerning repair of oxidative damage in mtDNA, the results had to be interpreted with caution because permanent transfection could have altered the genome of the cell in a manner that influenced the findings. To overcome this problem, the present experiments were initiated to find a "genetic switch" that would allow the control of individual gene products quantitatively and reversibly in a temporal manner. Among the several regulatory systems currently available, the tetracycline (Tet)-controlled system for the activation of transcription seemed best suited to fulfill the requirements of our study. Previously, this system has been used for conditional gene expression in mammalian cell culture (38), in transgenic plants (39), and in transgenic mice and other transgenic mammalian species (40, 41). HeLa cells were selected for this study because we previously discovered that these cells do not proficiently repair oxidative damage to their mtDNA (27), and

it has been reported that in these cells the Tet-systems exhibit no measurable "intrinsic leakiness" (28, 32). Repair of oxidative damage in the nucleus of these cells appears to be normal. This makes these cells preferable to cells in which OGG1 has been knocked out because those cells would be defective in both nuclear and mitochondrial repair. Although the reason that HeLa cells are defective in the repair of oxidative damage in mtDNA has yet to be fully elucidated, we believe a likely explanation is that there is a defect in the formation of the alternatively spliced transcript for mitochondrial localization. In support of this notion is our finding that HeLa cells have low levels of OGG1 protein in their mitochondria.

For targeting OGG1 to mitochondria we used the MTS from MnSOD, because previous work from our laboratory and the studies of others have found this to be a particularly strong MTS that effectively directs other proteins, including repair enzymes, to mitochondria (27, 33-35). We used the Tet-Off system, where the presence of Dox prevents transcriptional activation, for conditional expression of hOGG1 in mitochondria. This system was used because in the clones we isolated the amount of hOGG1 that could be produced was greater than with the Tet-On system. In the presence of Dox the exogenous recombinant MTS-OGG1 expression in mitochondria was low, and the cells behaved similarly in culture to the HeLa Tet-Off cell line, transfected with vector only.

Both the results from enzyme activity assays and DNA repair studies reveal that the Dox-dependent, expressed MTS-OGG1 is functional. The mitochondrial extracts isolated from MTS-OGG1 (Dox-) cells were markedly better able to cleave substrate than the same cells grown with Dox and vector only transfected cells. Mitochondrial DNA repair studies show that MTS-OGG1 (Dox-) cells were significantly more proficient at repairing oxidative damage in the mitochondrial genome. Combined with the viability studies, it can be concluded that recombinant OGG1 was conditionally expressed under the Dox-inducible promoter, and targeted to mitochondria in an active form. Furthermore, it enhanced the repair of oxidative damage to the sugar-phosphate backbone in mtDNA, and this augmented repair rendered the cells more resistant to oxidative stress. Thus, these findings support previous work from our laboratory using cells which constitutively overexpress OGG1 and provide a new opportunity for dissecting the various components of BER in mitochondria. Recently, much attention has been directed toward the identification and characterization of enzymes involved in BER of mtDNA. Initial evidence for a BER mechanism in mitochondria was provided by the isolation of a mammalian mitochondrial endonuclease that specifically recognizes AP sites and cleaves the DNA strand (36). Later, activities of an AP endonuclease, a DNA ligase III, and a DNA polymerase were identified in mitochondrial fractions from Xenopus oocytes (17). Additionally, a mitochondrial Oxidative Damage Endonuclease (mtODE) has been isolated from rat liver mitochondria (37). Recently, it was demonstrated that mtODE is a mitochondrial isoform of OGG1 (26). Although several of the enzymes involved in mtDNA BER have been identified, a thorough understanding of the exact roles these enzymes play is still incomplete. The results from the present study emphasize the crucial role that hOGG1 plays in the efficient repair of oxidative damage in mtDNA. Future

studies will be directed toward the conditional expression of other repair enzymes with glycosylase activity to other oxidative base lesions and lyase activity to better understand the importance of these activities in BER. Of particular interest is a second OGG activity, originally termed OGG2, which has been identified and partially characterized in HeLa cells (42). Also of immediate interest are two orthologs of MutM/Nei which have been identified in the human genome database and were originally named NEH1 and NEH2 (42). Both enzymes have since been renamed to NEIL1 and NEIL2. NEIL1 functions as a DNA glycosylase/AP lyase with broad substrate specificity (42), whereas NEIL2 has DNA glycosylase/AP lyase activity, which primarily recognizes oxidized cytosine derivatives (43).

The importance of mtDNA integrity for normal cellular homeostasis is only beginning to be appreciated. Because increased oxidative lesions are found in mtDNA in individuals with a variety of chronic diseases (1-9), regulated expression of mitochondrial repair enzymes may provide a beneficial gene therapeutic strategy for preventing or delaying the symptoms of these diseases. Additionally, conditional expression of repair enzymes may be a viable approach for protecting normal cells during cancer therapy, or sensitizing cancer cells to this treatment. This work represents some of the initial steps down this path.

FIGURE LEGENDS

Fig. 1. **The MTS-OGG1 Insert is Incorporated into HeLa Tet-Off Cells**.

A Southern blot was performed on total DNA isolated from seven HeLa Tet-Off clones transfected with the MTS-OGG1 construct, or a clone transfected with vector alone using the MTS-OGG1 sequence as a probe.

Fig. 2. **Dox-Regulated and Targeted Expression of OGG1 in Mitochondria**.

HeLa Tet-Off/ MTS-OGG1 transfected clones were cultured with or without 1 μg/ml Dox for four days to regulate OGG1 protein expression. Mitochondrial fractions were isolated and analyzed by Western blot analysis using anti OGG1 antiserum. Twenty μg of mitochondrial extracts isolated from vector and MTS-OGG1 transfected clones were loaded in each lane. Immunodetection of cytochrome C was performed to assure that the recombinant protein is in mitochondria.

Fig. 3. **Dox-Regulated OGG1 Activity in Mitochondria**.

Enzyme activity was measured using a labeled 24 -mer containing 8-OxoG. Mitochondrial fractions were isolated from vector and MTS-OGG1 transfected clones that had been maintained with and without Dox and the extracts incubated with labeled substrate. A significant increase of 9- mer cleavage product was observed in lanes containing extracts from MTC-OGG1 (Dox-) clones, indicating that OGG1 activity was significantly higher in those clones. The data represent three independent experiments.

Fig. 4. **Effect of Conditional Expression of OGG1 in Mitochondria on the Repair of Oxidative Damage to the Sugar-Phosphate Backbone in mtDNA**.

MTS-OGG1 and vector transfectants were treated with 800 μM menadione for 20 min and lysed immediately or allowed to repair for 2 or 6 h in normal culture media with or without Dox. Control cells were exposed to the drug diluent only. Total DNA was isolated from the lysed cells and quantitative alkaline Southern blot hybridizations with mtDNA specific probe were performed. **4A** is a representative autoradiograph. **4B** is a summation of the results of three independent experiments (values are the means $\pm$ SE of three separate experiments). An asterisk (*) indicates a significant difference ($p<0.001$).

Fig. 5. **OGG1 Dox-Regulated Expression and Cell Survival and Proliferation**.

Cells were maintained in the Dox $\pm$ media for four days and than were plated at low density into 60-mm dishes and allowed to adhere to the culture vessel for 24 h. They next were drugged with 25, 50, 75, or 100 μM menadione for 20 min and replenished with their normal media with or without Dox. Cells in cultures were allowed grow for ten days, at which time the cultures were fixed, stained with hematoxylin, and colonies counted. An average of the results $\pm$ SE from three separate clonogenic assays is shown. An

asterisk (*) indicates a significant difference ($p<0.001$).

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Figure 1

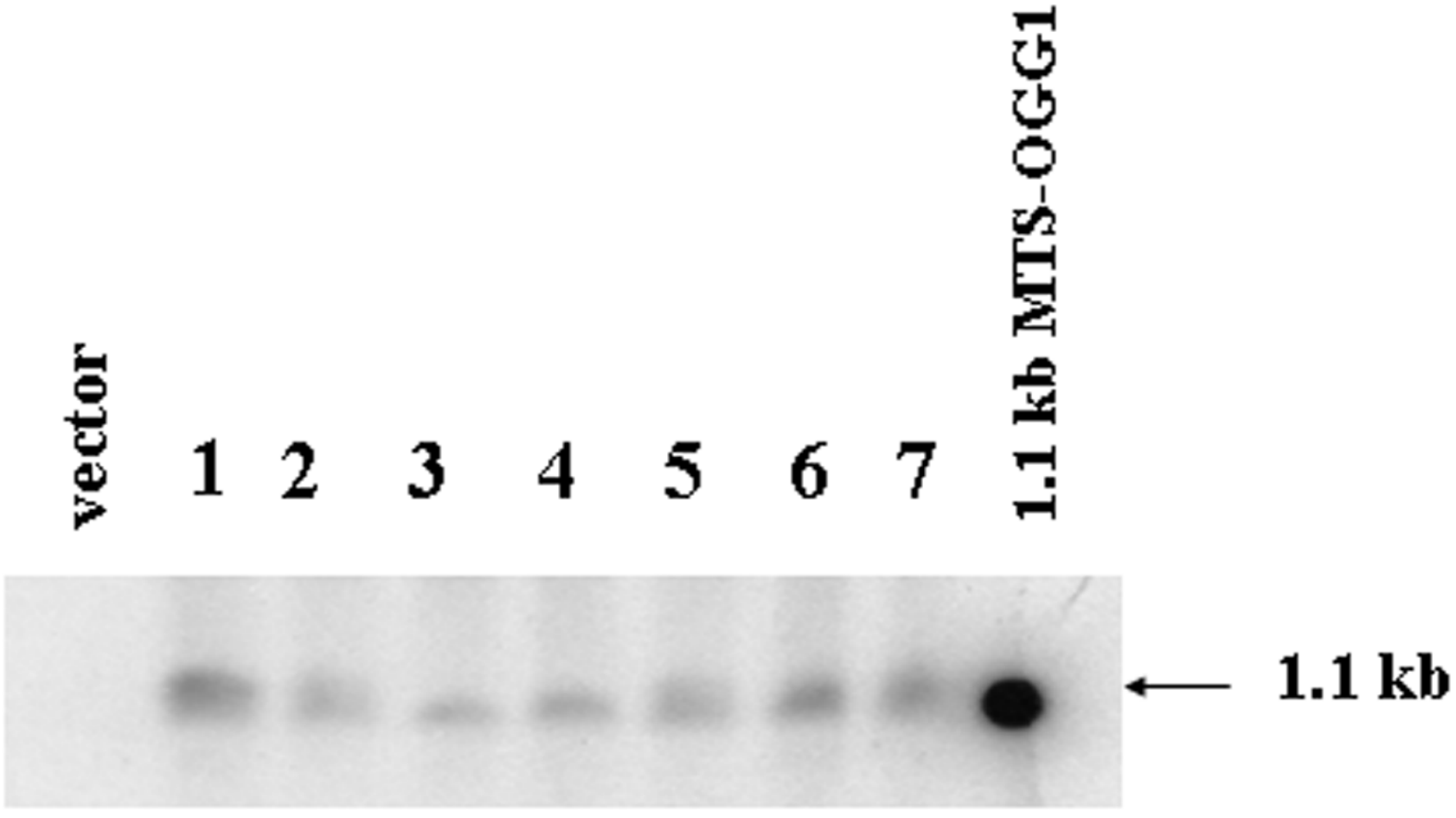
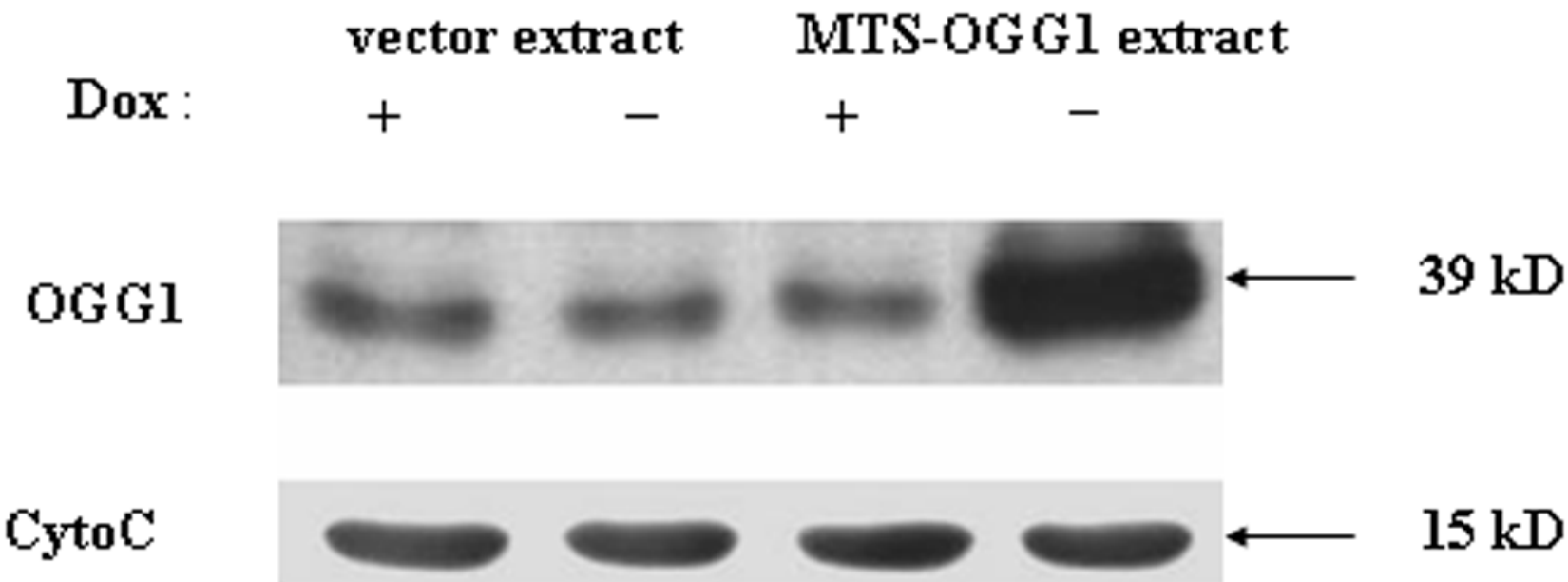


Figure 2



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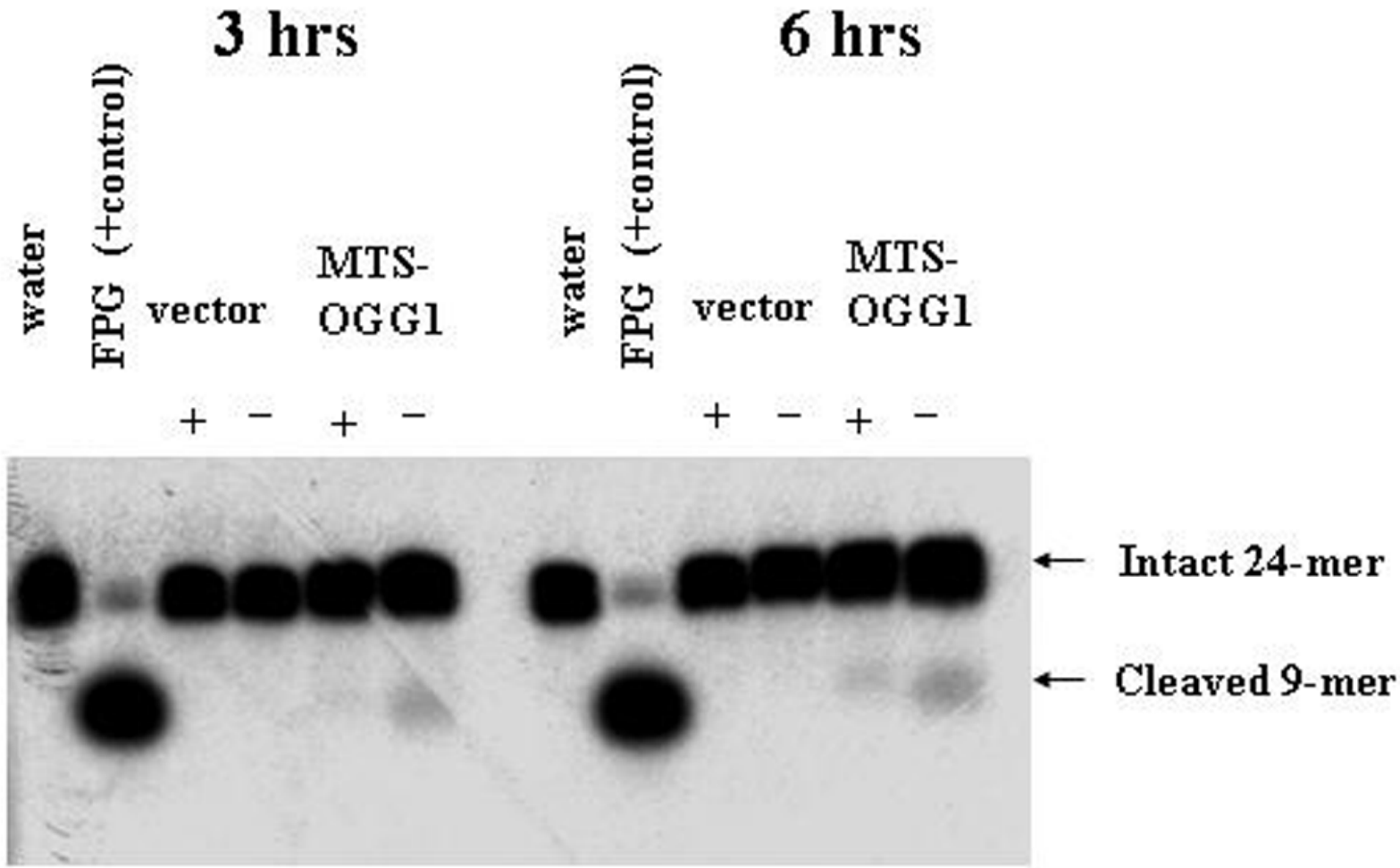
3 hrs
6 hrs
water
FPG (+control)
vector
MTS-OGG1
water
FPG (+control)
vector
MTS-OGG1
Dox:
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Intact 24-mer
Cleaved 9-mer

Figure 4A

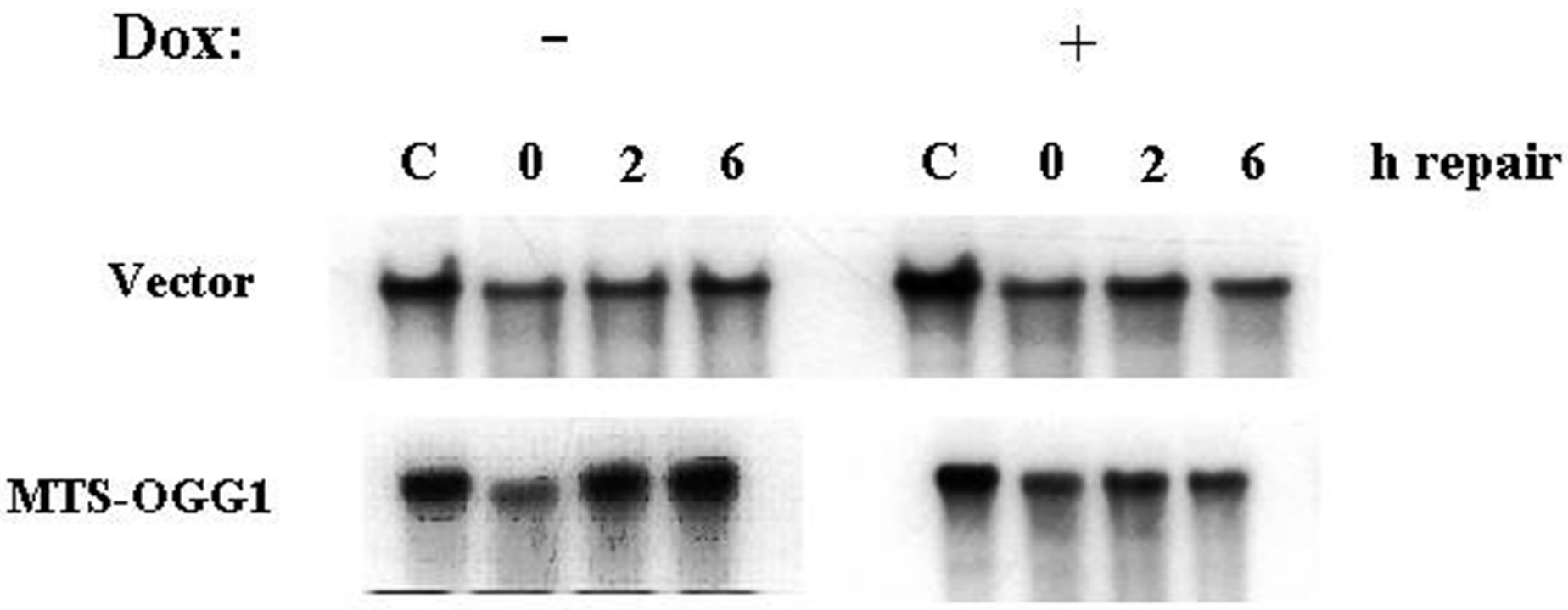
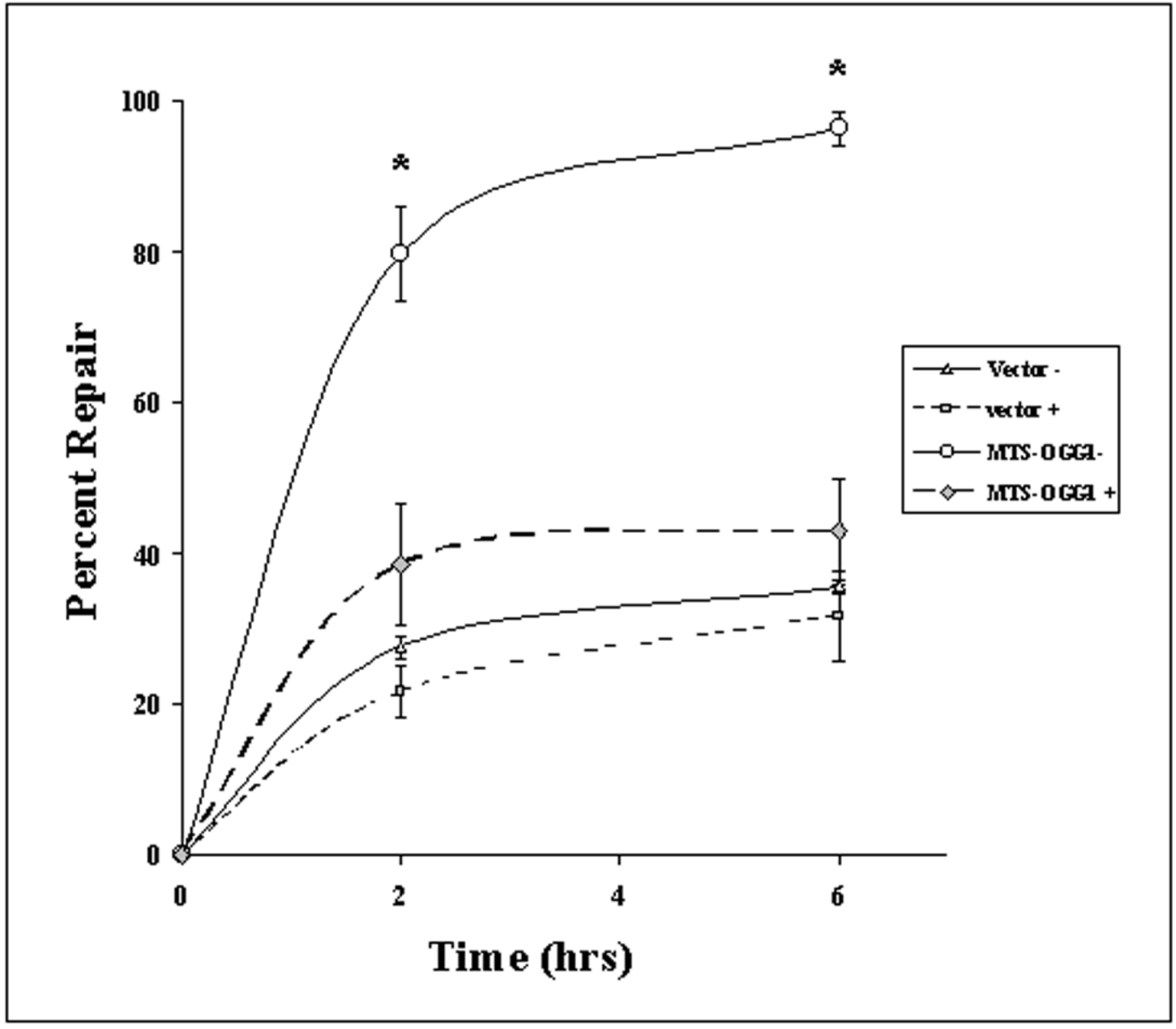


Figure 4B


Percent Repair
100
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60
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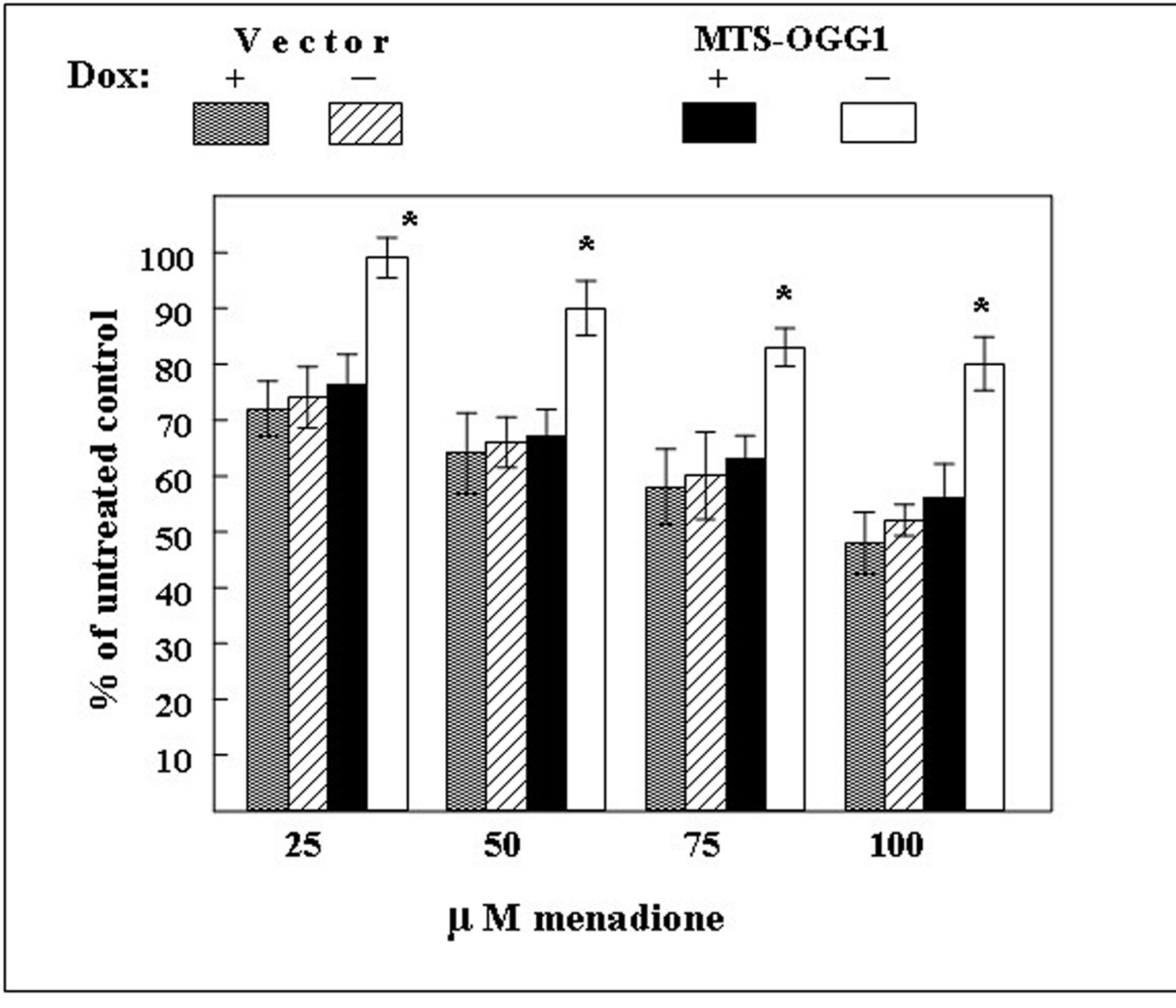
Figure 5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MTS-OGG1
Dox:
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% of untreated control
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90
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